Troubleshooting

Starting Out Right, Part IV — Additional Variables to Control Selectivity

Variables such as pH, temperature, and column type can be useful tools to help control peak spacing.

John W. Dolan
LC Troubleshooting Editor

Last month's installment of "LC Troubleshooting" described the role of solvent strength and solvent type in controlling selectivity in liquid chromatography (LC) separations (1). I explained that by making systematic variations in solvent strength, chromatographers can obtain dramatic changes in selectivity and retention. Changing the solvent type, from acetonitrile to methanol, for example, gave additional selectivity leverage during method development. In some cases, blending more than one organic solvent is useful.

Although varying solvent strength and type provides powerful tools for controlling the separation, these variables may be insufficient to obtain the desired resolution for a particular sample. For this situation, chromatographers must use other parameters to adjust selectivity. This month's "LC Troubleshooting" column will concentrate on three additional parameters: pH, temperature, and column type.

Mobile-Phase pH

For separations discussed in previous installments in this series, I suggested aqueous–organic solvent mobile phases in which the aqueous portion was water for neutral samples or low-pH buffer for ionic samples (1–3). Using low-pH mobile phases (pH 2–3) is a good starting point because these conditions suppress the ionization of both acidic compounds and the residual silanol groups on the silica surface. However, when ionic compounds are present, pH can have a marked influence on retention and selectivity, so if the initial low-pH mobile phase is unsatisfactory, the exploration of pH effects is a natural next step in method development.

Figure 1 illustrates the power of using pH to control selectivity (4). The figure shows that acids will be protonated, hydrophobic, and thus more strongly retained at low pH than at high pH. Conversely, bases will be neutral and well retained at high pH, whereas low pH results in ionization and poor retention in a reversed-phase system. Neutral compounds are unaffected by pH, so the retention will vary little, if at all, when the mobile-phase pH is changed.

Figure 1 also illustrates that a mixture of acids, bases, and neutrals will cause many retention reversals when the pH is changed. This effect can be a powerful tool to control selectivity and obtain a separation impossible through the adjustment of other parameters, but it also indicates that chromatographers must take care to control the pH and avoid any unintended changes in selectivity.

The plot of retention versus pH for compound 4, which is a base, in Figure 1 shows a shape closely resembling a titration curve. Not surprisingly, the midpoint of this curve is at the pK_a for this compound, which is

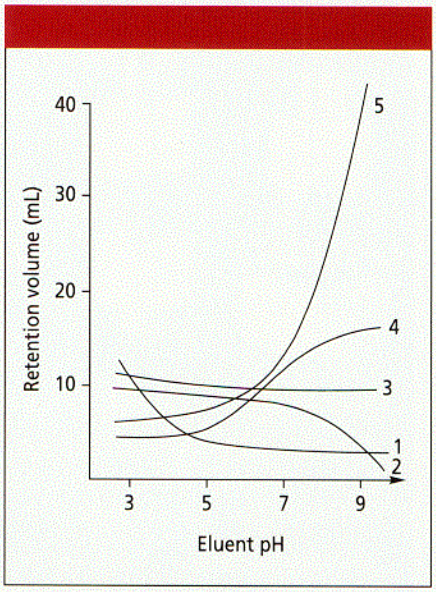


Figure 1: The influence of pH on retention of a mixture of acids (curves 1 and 2), bases (curves 4 and 5), and neutral (curve 3) compounds. (Reprinted from reference 4 with permission.)

approximately pH 7. Acids show the same curve shape in mirror image, although the pH range in Figure 1 is insufficient to show the entire curve for any compound except number 4.

Figure 1 shows that an adequate separation of all sample components can be obtained at pH 5 or 7. However, at pH 7, the method is much more likely to be sensitive to small changes in pH, particularly for compounds 4 and 5. For maximum method robustness, it is best to work at a pH at which the compound or compounds of interest are either fully ionized or ionization is suppressed. This condition means that chromatographers should operate at more than ±1.5 pH units from the pK_a value. As usually is the case, workers may need to compromise for the best results, but operation at pH 5 will be less sensitive to pH changes than that at pH 7 for this sample.

As mentioned earlier, I recommend starting the initial run with most ionic samples at low pH; for example, pH 2.5. Under these conditions, adjust the organic solvent content of the mobile phase to obtain a good retention range for neutral and nonionized samples. To screen for gross effects of pH, chromatographers can change the mobile-phase pH while keeping the organic solvent at a constant concentration. For example, make the initial run at pH 2.5, then change to pH 7.0 for another run to see if dramatic changes occur. However, to fine-tune the mobile-phase pH, it is best to vary the mobile phase in steps not exceeding 0.5 pH units because retention and peak spacing can change dramatically with small changes in pH. These dramatic changes can make peak tracking difficult, especially if peak areas change, which can happen with pH changes.

Figure 2 shows an example of fine-tuning the mobile-phase pH for a group of benzoic acids (5). In each case, only the pH is varied. Whenever examining a set of chromatograms such as in this figure, it is useful to concentrate on the critical (least resolved) peak pairs. Peaks 2, 6, and 9 move dramatically in relation to the other peaks in the sample. A satisfactory separation of all nine components can be obtained at pH 3.0. As is expected with a group of organic acids, lower pH will reduce the degree of ionization and make the components more hydrophobic and thus more strongly retained.

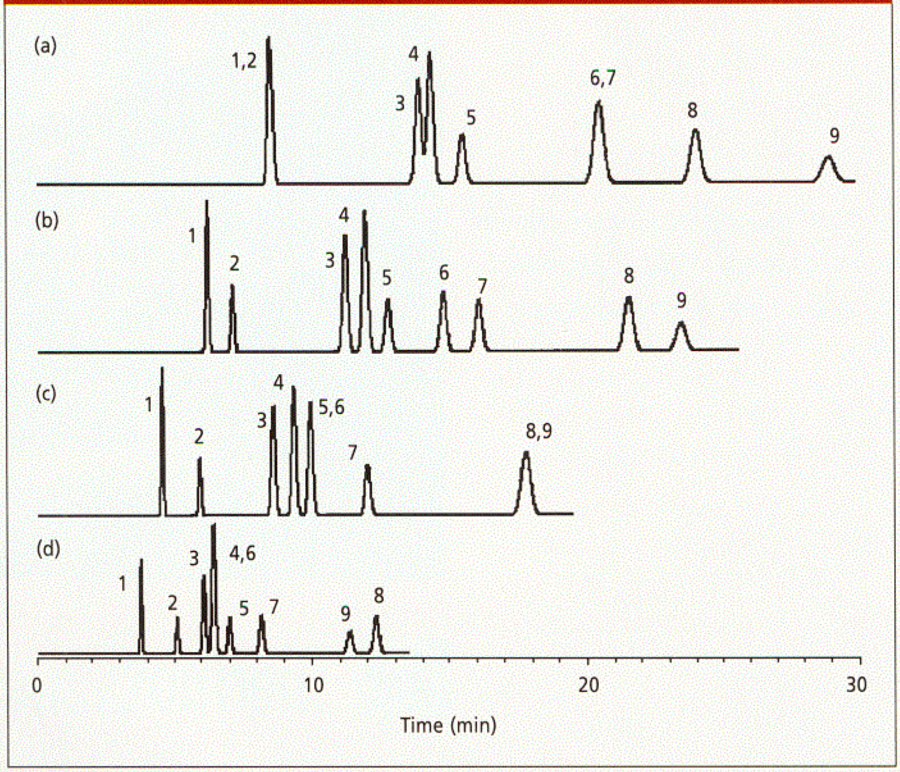


Figure 2: Simulated separation of nine benzoic acids at (a) pH 2.5, (b) pH 3.0, (c) pH 3.5, and (d) pH 4.0. Peaks: 1 = 2-nitrobenzoic acid, 2 = phthalic acid, 3 = impurity, 4 = 2-fluorobenzoic acid, 5 = 3-cyanobenzoic acid, 6 = 2-chlorobenzoic acid, 7 = 3-nitrobenzoic acid, 8 = 3-fluorobenzoic acid, 9 = 2,6-dimethylbenzoic acid. The conditions are listed in Table I. The simulated chromatograms were generated from the data of reference 5.

When using pH to control an LC separation, it is especially important to obey the fundamental rules of buffer usage. Remember that a buffer is effective in the range of ±1 pH unit from the pK_a of the buffer. Outside this range, buffering is marginal, meaning that methods using those conditions will be less robust. This rule means that phosphate buffer is a good choice in the pH 2.0–3.1 and 6.2–8.2 regions (below pH 2, bonded-phase stability is a problem), whereas acetate buffer is a better choice in the pH 3.8–5.8 region. Although the ideal buffering ranges for these two buffers don't overlap, stretching the range of each buffer by less than one-half a unit will allow a blend of acetate and phosphate to make a buffer useful throughout the entire pH 2–8 range.

Column Temperature

Another variable that can provide useful changes in selectivity is column temperature. Most workers consider temperature only in terms of retention time changes. As a general rule, retention in reversed-phase LC can be expected to change 1–3% for each 1 °C change in temperature. Figure 3 illustrates this rule; in this figure retention changes roughly 25% (19 min to 25 min for the last peak) with a 15 °C change in temperature, or approximately 1.7%/°C. Table I lists the conditions for this separation. Temperature control is important for reproducible retention times.

It is less appreciated that changes in temperature can cause changes in selectivity in LC separations. This condition is illustrated in Figure 3 for the same benzoic

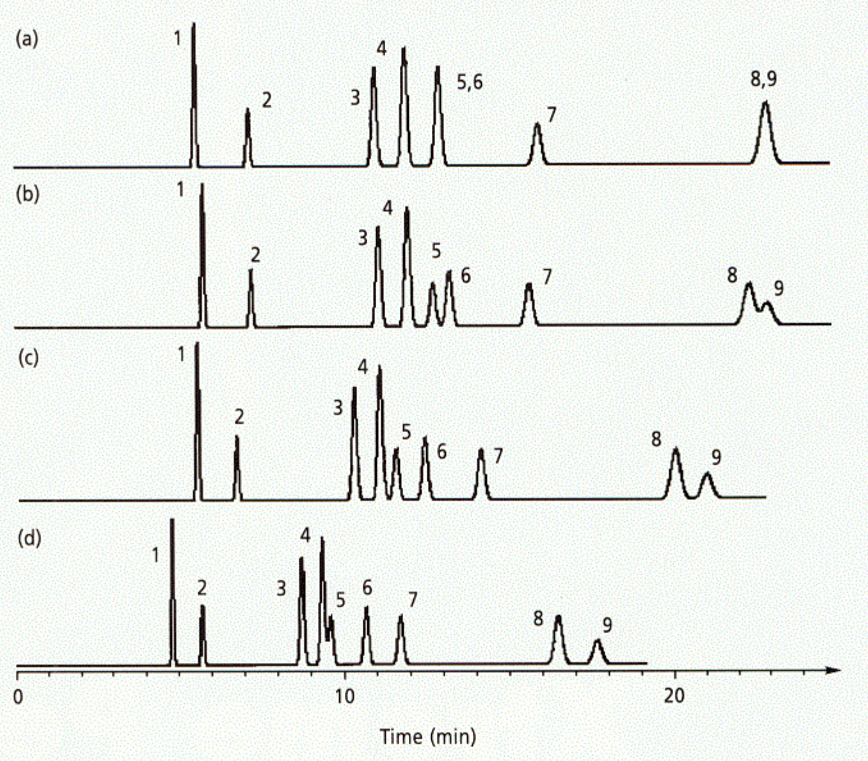


Figure 3: Simulated separation of nine benzoic acids at (a) 30 °C, (b) 35 °C, (c) 40 °C, and (d) 45 °C. Peaks: Same as in Figure 2. The conditions are described in Table I. The simulated chromatograms were generated from the data of reference 5.

Table I: Conditions for Figures 2 and 3

Variable	Figure 2	Figure 3
Column	250 mm × 4.6 mm, 5 µm d_p C8	Same
Flow rate	1 mL/min	Same
Methanol (%)	35%	Same
Buffer	25 mM phosphate	Same
pH	Varies	pH 3.2
Temperature	35 °C	Varies

acid sample that was shown in Figure 2. Although the general trend is shorter retention times with increased column temperature, important selectivity changes take place, too. Notice the movement of peak 5 as the temperature is increased, changing from coelution with peak 6 at 30 °C to beginning to merge with peak 4 at 45 °C. Because peaks move in a regular fashion with changes in mobile-phase conditions, peaks 4 and 5 would completely merge when the temperature is increased to higher than 45 °C (overlap is complete at 53 °C). Similarly, peaks 8 and 9 move relative to each other when the column temperature is changed. For this sample, a temperature of 40 °C will provide baseline separation of all sample components.

Why does temperature provide such dramatic changes in selectivity for this sample? In this case, the changes to a large degree are due to the ionic character of the sample. The temperature will influence the ionization characteristics, hydrophobic retention of ionized versus nonionized compounds, silanol interactions with ionic species, mobile-phase pH, and the pK_as of sample components. With all of these influences, it would be surprising if a change in temperature *did not* influence selectivity.

The practical result for this benzoic acid sample is that workers can obtain a satisfactory separation under more than one set of conditions. The similarity between the pH 3.0 (35 °C) run of Figure 2b and 40 °C (pH 3.2) run of Figure 3c is striking. Both conditions provide baseline separation of all components in approximately 25 min. When ionic species are present in the sample, column temperature and mobile-phase pH will play an important role in peak spacing.

Column Selectivity

Up to this point in the discussion, I have talked about changes in selectivity accomplished by making changes in the mobile-phase composition. This approach usually is the most convenient and least expensive way to change selectivity. An alternative approach, usually best left until mobile-phase alternatives have been exhausted, is to rely on changes in column selectivity to control the separation. As with changes in mobile-phase selectivity, chromatographers should make significant changes in column selectivity to take maximum advantage of this parameter. Therefore, changing from one brand of C18 column to another or from a C18 to a C8 column will not be very fruitful. These kinds of changes may result in selectivity variations, but they tend to be minor when compared with changes in the stationary-phase chemistry.

For reversed-phase separations, three column types — C18 or C8, cyano, and phenyl — traditionally are selected. Figures 4 and 5 illustrate the changes in selectivity that occur between a C8, cyano, and phenyl bonded phase for two different samples. The sample of Figure 4 (6) is a group of substituted benzoic acids, some of which are in the sample of Figures 2 and 3. The most prominent change is in the retention order of the 4-5-6 triplet, in which compound 4 comes out first with the C8 column, but last with the other two. Also, the peak spacing between peaks 1 and 2 changes markedly.

An herbicide mixture was used for the separations of Figure 5 (6). Peaks 1 and 2 run together on the cyano column and the separation of 6 and 7 is reduced as well. No retention order changes are observable for the herbicide mixture on these columns.

It is apparent that column chemistry changes may yield dramatic selectivity changes, as is the case for the benzoic acids in Figure 4, or the changes may be much more subtle, as with the herbicides in Figure 5. It is impossible to accurately predict changes in selectivity between columns, and no software is available to assist in fine-tuning selectivity changes such as the software used for mobile-phase modifications, so column changes tend to be trial-and-error in nature. For this reason, I recommend trying the easier and often more effective mobile-phase changes before exploring column selectivity.

In my discussions with other users, I have found that the phenyl column is used infrequently today as a tool to enhance selectivity. In the last few years, the polar embedded phases have come into favor. These phases tend to be C18 or C8 phases with an amine-containing polar function attached near the base of the C18 chain, typically on the third carbon from the base. The result is bonded phases that often have distinctly different selectivity characteristics than traditional C18 or C8 phases. In my laboratory, we have found these phases to be a good third member to go with the C18–C8 and cyano phases when exploring column selectivity. Some brand names are Discovery Amide

(Supelco, Bellefonte, Pennsylvania), Symmetry Shield (Waters Corp., Milford, Massachusetts), and Zorbax Bonus RP (Agilent Technologies, Wilmington, Delaware). Check with your favorite column supplier for other columns.

Other Variables

The discussion thus far has concentrated on the variables that will give the most leverage in changing the selectivity of a separation. Many other variables can be explored. For example, ion-pair reagents often are used to enhance selectivity, especially when a mixture of acids and bases is present in the sample, but ion pairing tends to be more problematic than simple pH control. Chromatographers can exploit special chemical characteristics of the sample — such as the presence of chelators, chiral compounds, or shape differences — to gain selectivity. When a good effort has been made at obtaining a separation with the traditional reversed-phase variables, workers may want to explore modes of chromatography such as ion exchange, normal phase, or size exclusion. Reference 7 is a good resource for method development for these and other samples.

Conclusions

By the time chromatographers have explored mobile-phase solvents, pH, temperature, column type, and perhaps ion pairing, they likely will have obtained a satisfactory reversed-phase separation. Sometimes the selectivity, or peak spacing, is satisfactory, but the peaks still are not quite baseline separated. In other cases, the peaks may be overseparated, which is a sign of wasted time. This is the stage in the method development process when it is appropriate to see if changes in the column size, mobile-phase flow rate, or packing particle diameter can be used to improve the results. These changes in column parameters will be the subject of next month's "LC Troubleshooting" column.

References

(1) J.W. Dolan, *LCGC* **18**(2), 118–125 (2000).
(2) J.W. Dolan, *LCGC* **17**(12), 1094–1097 (1999).
(3) J.W. Dolan, *LCGC* **18**(1), 28–32 (2000).
(4) P.J. Twitchett and A.C. Moffat, *J. Chromatogr.* **111,** 149 (1975).
(5) J.W. Dolan, D.C. Lommen, and L.R. Snyder, *J. Chromatogr.* **535,** 55–74 (1990).
(6) J.J. DeStefano, J.A. Lewis, and L.R. Snyder, *LCGC* **10**(2), 130–139 (1992).
(7) L.R. Snyder, J.J. Kirkland, and J.L. Glajch, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1997).

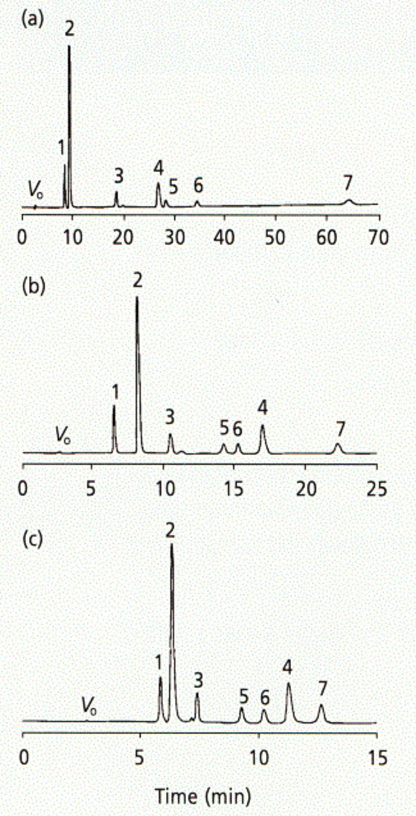


Figure 4: Separation of a substituted benzoic acid sample using 250 mm × 4.6 mm (a) C8, (b) phenyl, and (c) cyano columns. Mobile phase: (a) 25% methanol–buffer, (b) 35% methanol–buffer, (c) 35% methanol–buffer. Peaks: 1 = phthalic acid, 2 = 2-nitrobenzoic acid, 3 = 2-fluorobenzoic acid, 4 = 3-nitrobenzoic acid, 5 = 2-chlorobenzoic acid, 6 = 3-fluorobenzoic acid, 7 = *m*-toluic acid. (Reprinted from reference 6 with permission.)

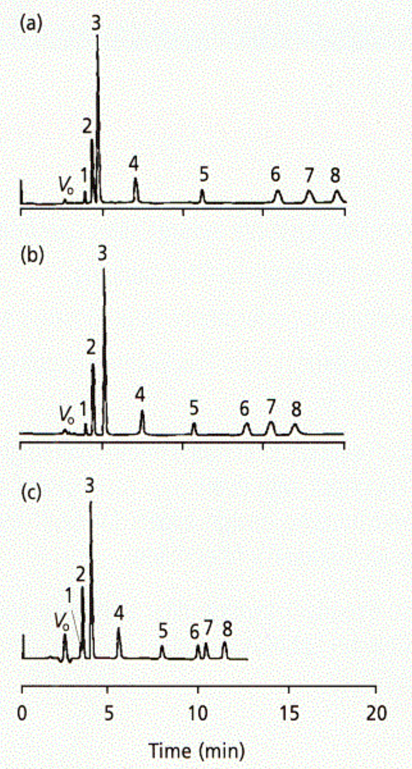
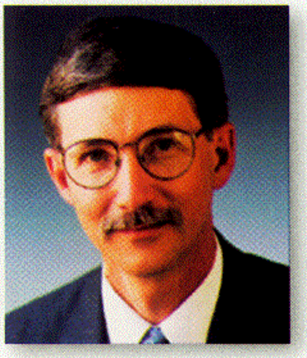


Figure 5: Separation of an herbicide mixture using a 40% acetonitrile–water mobile phase on (a) C8, (b) phenyl, and (c) cyano columns. Peaks: 1 = atrazine, 2 = metribuzin, 3 = fenamiphos sulfoxide, 4 = fenamiphos sulfone, 5 = diuron, 6 = propanil, 7 = propanamide metabolite, 8 = swep. (Reprinted from reference 6 with permission.)



John W. Dolan

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of LCGC's editorial advisory board. Direct correspondence about this column to "LC Troubleshooting," LCGC, 859 Willamette Street, Eugene, OR 97401, e-mail John.Dolan@LCResources.com.

For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at www.chromforum.com.